Autonomic Neuroscience: Basic and Clinical 187 (2015) 56–62

Contents lists available at ScienceDirect

Autonomic Neuroscience: Basic and Clinical

journal homepage: www.elsevier.com/locate/autneu

# *N*-acetylmannosamine improves sleep–wake quality in middle-aged mice: Relevance to autonomic nervous function

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ARTICLE INFO

*Article history:*
Received 12 June 2014
Received in revised form 22 August 2014
Accepted 11 November 2014

*Keywords:*
Aging
Autonomic nervous system
Body temperature
Heart rate
*N*-acetylmannosamine
Orexin
Sleep

ABSTRACT

Aging is associated with a variety of physiological changes originating peripherally and centrally, including within the autonomic nervous system. Sleep–wake disturbances constitute reliable hallmarks of aging in several animal species and humans. Recent studies have been interested in *N*-acetylmannosamine (ManNAc) a potential therapeutic agent for improving quality of life, as well as preventing age-related cognitive decline. In this study, ManNAc (5.0 mg/ml) was administered in the drinking water of middle-aged male C57BL/6J mice (55 weeks old) for 7 days. Mice were housed under a 12:12 h light:dark cycle at 23–24 °C. We evaluated bio-behavioral activity using electrocardiogram, body temperature and locomotor activity recorded by an implanted telemetry transmitter. To estimate sleep–wake profile, surface electroencephalogram and electromyogram leads connected to a telemetry transmitter were also implanted in mice. Autonomic nervous activity was evaluated using power spectral analysis of heart rate variability. ManNAc-treated mice spent more time in a wakeful state and less time in slow wave sleep during the dark phase. Parasympathetic nervous activity was increased following ManNAc treatment, then the sympatho-vagal balance was shifted predominance of parasympathetic nervous system. Furthermore, improvement in sleep–wake pattern was associated with increased parasympathetic nervous activity. These results suggest that ManNAc treatment can improve bio-behavioral activity and sleep–wake quality in middle-aged mice. This may have implications for improving sleep patterns in elderly humans.


## 1. Introduction

The advent of population aging has led to an increase in age-related health issues. Many age-related diseases can be alleviated and delayed by adopting an active and happy lifestyle. Aging is associated with a variety of physiological alterations peripherally and centrally including within the autonomic nervous system.

Endogenous sialic acid is limited in aging organisms as suggested by several reports on reduced sialylation levels in the aged (Saito et al., 2002; Uslu et al., 2004; Sprenger et al., 2009). Sialic acids are the most abundant terminal monosaccharides on glycoconjugates of eukaryotic cell surfaces and are involved in a variety of cellular functions. Sialylated glycosphingolipids and glycoproteins play important roles in cell contact, communication, and signaling processes (Allende and Proia, 2002; Hakomori, 2002; Schnaar, 2004; Varki, 2007). In the nervous system, gangliosides play a major role in synaptic contacts, neuronal plasticity, recovery of neuronal function, and neuron–glia stability. Further, brain ganglioside loss is observed with the old age (Kuracun et al., 1991).

*N*-acetylmannosamine (ManNAc), the physiological sialic acid precursor, is converted to *N*-acetylneuramine acid (NeuNAc), the most abundant sialic acid. As ManNAc is converted to NeuNAc after delivery into cells, it is potentially a rich source of sialic acid *in vivo*. Although the challenge in administering sialic acid *in vivo* is mainly its rapid excretion in the urine, it is suggested that the intragastric route may be more advantageous in increasing the serum abundance of sialic acid (Malicdan et al., 2009). Furthermore, ManNAc has recently attracted interest as a potential therapeutic agent to improve quality of life in addition to cognitive function, because ManNAc treatment alleviated the age-related decline in learning ability and memory in both mice and dogs (Kikusui et al., 2012; Nagasawa et al., 2014). In addition, ManNAc, but not NeuNAc, treatment promotes the generation of orexin neurons from mouse embryonic stem (ES) cells *in vitro* (Hayakawa et al., 2013).

The autonomic nervous system is essential for fast adaptation or modulation of visceral functions during changes in external and internal environments. There are significant age-related changes in autonomic nervous activity (Hotta and Uchida, 2010). Heart rate variability (HRV) has gained increasing interest as a noninvasive index of autonomic nervous activity (Malik et al., 1996). A number of methods for the assessment of HRV, such as time-domain and frequency-domain

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http://dx.doi.org/10.1016/j.autneu.2014.11.005
1566-0702/

measures, have been proposed. We have previously established power spectral analysis of HRV in many animal species including diurnal variation of autonomic nervous activity (Kuwahara et al., 1994, 1996, 1999; Ishii et al., 1996b; Hashimoto et al., 1999; Kawaguchi et al., 2005).

We speculated that there is a link between available sialic acid levels and the decline of neuronal functions observed with increasing age as outlined above. Aging has been associated with poor bio-behavioral activity and sleep quality, therefore we hypothesized that ManNAc treatment might improve these factors in middle-aged mice. Because heart rate (HR), body temperature (BT), and locomotor activity (LA) represent physiological and pathophysiological conditions, these parameters are generally monitored as a useful index of bio-behavioral activity. In this study, we assessed the impact of ManNAc on bio-behavioral activity and sleep quality in middle-aged mice, by adding it to the drinking water for 7 days. Moreover, many studies, in a variety of experimental and clinical conditions, have used HRV; therefore, we used this method to assess autonomic nervous activity. Accordingly, we measured 24-h patterns of bio-behavioral activity and sleep–wake patterns in middle-aged mice, and autonomic nervous activity involved in regulating these functions.

## 2. Methods

### 2.1. Animals

Young (12 weeks old; n = 4), middle-aged (55 weeks old; n = 10), and old (95 weeks old; n = 3) male C57BL/6J (B6) mice were used. Mice were individually housed in conventional cages with 12:12 h light:dark photoperiod (lights on at 0800), in a temperature controlled room (23–24 °C). Standard mouse chow (MF; Oriental Yeast, Tokyo, Japan) and water were supplied *ad libitum*. All animal experiments were performed in accordance with the Institute of Ethical Guidelines under the protocols approved by the Animal Experimental Expert Committee of the University of Tokyo.

### 2.2. Implanting the transmitter for ECG recording

A telemetric transmitter for electrocardiogram (ECG) (TA10ETA-F20, Data Sciences International, St. Paul, MN, USA) was surgically implanted under pentobarbital sodium anesthesia (40 mg/kg, i.p.) at the cervical subcutaneous region. The paired wire electrodes, in a precordial bipolar lead (Apex–Base lead), were secured subcutaneously (Ishii et al., 1996a). The parameters measured were HR, BT, and LA. LA was measured and the number of counts across the grids of a signal-receiving board was analyzed (RPC-1, Data Sciences International St. Paul, MN, USA) during a 1 min period. Animals were allowed to recover and habituate to recording conditions for 10 days prior to the start of the experiment.

### 2.3. Implanting the transmitter for sleep analysis

Mice were anesthetized with pentobarbital sodium (40 mg/kg, i.p.), and surface electroencephalogram (EEG) and electromyogram (EMG) leads connected to a telemetric transmitter (F40-EET, Data Sciences International, St. Paul, MN, USA) were implanted to record vigilance states. To secure the leads, two stainless-steel screws (diameter, 1.0 mm) were attached to the skull. The positions of the bilateral EEG leads were determined using a mouse brain atlas (caudal of bregma 1 mm, lateral of bregma 1 mm (right and left hemispheres)), and muscular leads were inserted into the temporalis muscles. The entire assembly was anchored to the skull with dental cement. The transmitter was placed temporalis in a blunt dissected channel across the animals back. Animals were allowed to recover and habituate to recording conditions for 10 days prior to the start of the experiment.

### 2.4. Data recording

Individually caged mice were placed on a signal-receiving board (RPC-1, Data Sciences International St. Paul, MN, USA) and housed in a light and temperature controlled chamber (MIR-553, Sanyo, Tokyo, Japan). The EEG–EMG recording system includes the implanted transmitter with EEG–EMG leads, which transmit bioelectric signals (EEG and EMG) by radiotelemetry to the receiver (PhysioTel RPC-1, Data Sciences International, St. Paul, MN, USA). EEG signals (1.0–50.0 Hz bandpass) and EMG signals (10–50.0 Hz bandpass) were amplified, filtered, recorded, digitized and stored electronically on a computer using a Data Exchange Matrix and Dataquest A.R.T. 4.1 software (Data Sciences International). The EEG and EMG signals were shown on the computer monitor during the recording sessions. The signals of BT and LA were also recorded continuously every 5 min with the Dataquest A.R.T. 4.1 analyzing system.

### 2.5. Experimental design

ManNAc (Sanyo Fine Co. Osaka, Japan) was dissolved in water at a concentration of 5.0 mg/ml. Each signal was recorded for 7 days as a control and ManNAc was administered in drinking water for 7 days. The dose was selected according to the previous study (Kikusui et al., 2012).

### 2.6. Determination of behavioral states

To score sleep–wake behavioral states, EEG and EMG signals were visualized with Neuroscore software (Data Sciences International) and sleep–wake behaviors were then scored manually on a personal computer. Specifically, consecutive 10 s epochs of EEG and EMG signals were graphically shown on the computer monitor. Following this, EEG–EMG data were classified into one of the following four behavioral states based on the EEG and EMG signals: (1) slow wave sleep (SWS, spindling and high-voltage EEG with slow waves, low-voltage EMG); (2) rapid eye moving (REM) sleep (low-voltage and fast EEG combined with EMG activity approximately 50% lower amplitude than that observed in SWS, with occasional short-duration, large-amplitude deflections due to muscle twitches); (3) quiet wake (QW: low-voltage fast EEG with EMG activity of a mean amplitude twice that observed in SWS); and (4) active wake (AW: low-voltage fast EEG, sustained high-voltage EMG of approximately twice that observed in QW, with frequent movement deflections).

### 2.7. Power spectral analysis of heart rate variability (HRV)

ECG signals were also continuously recorded and analyzed by an ECG processor (SBP2000, Softron, Tokyo, Japan) continuously. An off-line power spectral analysis of HRV was performed on an ECG processor (SRV2W, Softron, Tokyo, Japan) using the recorded ECG data. HRV was calculated using the detected R waves. Data sets of 512 points were resampled at 70 ms. A Hamming window was applied to each dataset and following this, datasets underwent fast Fourier transform to obtain the power spectrum of the fluctuation. The frequency range was used according to a previous study (Ishii et al., 1996b), with low frequency (LF) classed as between 0.1 and 1.0 Hz and high frequency (HF) classed as between 1.0 and 5.0 Hz. HF and LF power corresponded to the parasympathetic nervous activity, and both sympathetic and parasympathetic nervous activities, respectively (Malik et al., 1996).

### 2.8. Statistical analyses

Data are expressed as mean ± SEM. Paired *t*-tests were used to compare parameters. $P < 0.05$ was considered significant.

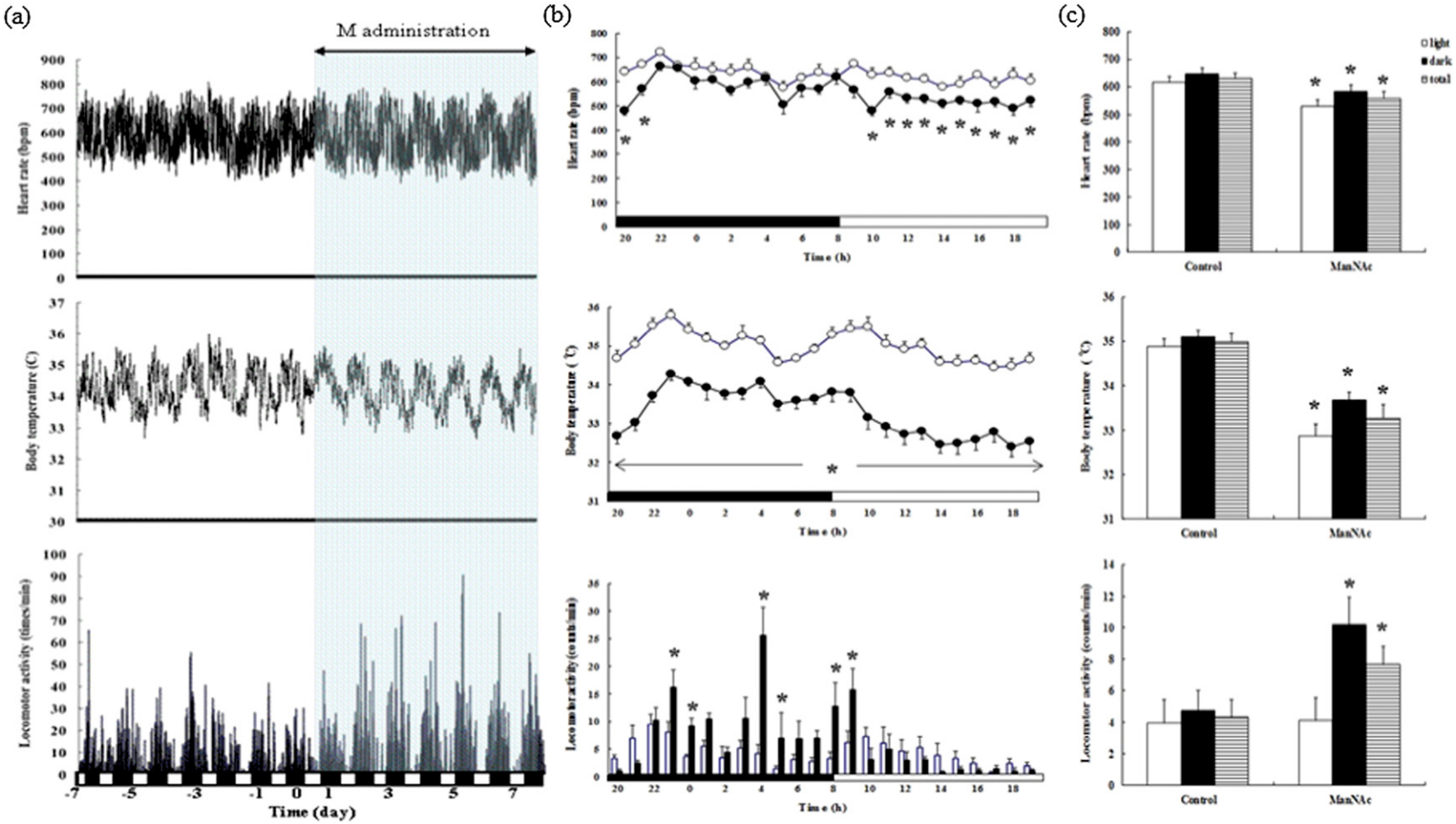


**Fig. 1.** Effects of ManNAc on bio-behavioral activity in middle-aged mice. (a) Representative changes of heart rate, body temperature, and locomotor activity with ManNAc (M) administration in a middle-aged mouse. (b) Hourly heart rate, body temperature, and locomotor activity for 24 h in the control period (open circle and bar) and during ManNAc treatment (filled circle and bar). Data represent mean ± SEM (n = 5). *Significantly ($P < 0.05$) different from control values. (c) Light- and dark-phase heart rate, body temperature and locomotor activity in the control period and during ManNAc treatment. Twelve-hour values for each mouse in each period are taken and averaged to get an average per mouse and then are summarized for 5 mice to get a mean ± SEM for each of the periods. *Significantly ($P < 0.05$) different from control values.

## 3. Results

### 3.1. Effects of ManNAc on bio-behavioral patterns

First, we evaluated the efficacy of ManNAc as a treatment to alter bio-behavioral activity in young, middle-aged, and old mice. Representative traces of HR, BT and LA 7 days prior to and during ManNAc (5 mg/ml) treatment in middle-aged mice are shown in Fig. 1a. HR and BT were decreased 2 days after ManNAc treatment in middle-aged mice. Conversely, LA was increased during ManNAc treatment. Changes in 24-h plots of these parameters are shown in Fig. 1b and the values for the light and dark phases prior to control and following 7 days of ManNAc administration are summarized in Fig. 1c. Diurnal (nocturnal) patterns, in which the values of these parameters during the dark phase (2000–0800) were higher when compared to those during the light phase (0800–2000), became much more prominent with ManNAc treatment. HR in ManNAc-treated mice was significantly lower during 0900–2100 when compared with the control period. BT in ManNAc-treated mice was significantly lower throughout the whole day when compared with control condition. In addition, LA in ManNAc-treated mice during the dark phase was significantly higher when compared with the control period. However, there was no significant effect of ManNAc treatment on bio-behavioral activity in both young and old mice (Table 1).

### 3.2. Cumulative time spent in sleep–wake states

Following 7 days of ManNAc treatment, middle-aged mice had a significantly reduced total sleep time when compared with control (ManNAc *vs* control: 994 ± 31 *vs* 1180 ± 46 min, $P < 0.05$). Reduced total sleep time in ManNAc-treated middle-aged mice was due to less time spent in SWS (57.1 ± 1.1% and 71.6 ± 1.9%, respectively, $P < 0.05$) although REM sleep was slightly increase by ManNAc treatment (12.0 ± 0.9% and 9.9 ± 0.8%, respectively). The percentage of time spent in QW did not differ over 24 h with ManNAc treatment; therefore, we did not consider AW and QW separately, and analyzed wakefulness (WAKE) (AW + QW). ManNAc-treated middle-aged mice spent significantly more time in WAKE when compared with control during 24-h recording (30.8 ± 0.9% *vs* 18.5 ± 1.1%, $P < 0.05$).

Time spent in WAKE and SWS was not significantly different between control and ManNAc-treated middle-aged mice during the light phase (Fig. 2a). In contrast, ManNAc-treated middle-aged mice spent significantly more time in WAKE, and less time in SWS, during the dark phase ($P < 0.05$, Fig. 2a). Thus, total time spent in sleep was also significantly less and time spent in WAKE was greater in ManNAc-treated middle-aged mice compared with control. These differences were mainly dependent upon changes that occurred in the dark phase. The wakefulness ratio between the 12-h light and dark phases (an indicator of diurnal variation in wakefulness) was significantly lower following ManNAc treatment when compared with control, suggestive of the high diurnal amplitude of the wakefulness rhythm in ManNAc-treated middle-aged mice.

As illustrated in Fig. 2b, a detailed analysis of sleep–wake parameters at 1-h intervals across the 24-h recording period indicated that ManNAc-treated middle-aged mice spent significantly less time in SWS during 2000–2200 and 0700 and significantly more time in WAKE during the 1800–2200, 0100–0300, 0700–0800 and 1800 when compared with control ($P < 0.05$ for all comparisons, Fig. 2b). The 1-h analysis also indicates that differences observed during the 24-h recording period were primarily due to differences in the vigilance states during the dark phase.

### 3.3. Autonomic nervous activity

The values of HRV parameters for the light and dark phases during the control period and following 7 days of ManNAc administration in middle-aged mice are summarized in Fig. 3. In the control period, HF values were almost the same between the light and dark phases, and LF/HF ratio in the light phase was higher than that in the dark phase. ManNAc-treated middle-aged mice had significantly higher HF power throughout the day and a lower LF/HF ratio in the light phase when compared with the control period. These results indicated that ManNAc enhanced parasympathetic nervous activity.

### 3.4. Correlation of sleep–wake state with parasympathetic nervous activity

Correlation between sleep–wake state and parasympathetic nervous activity was analyzed using mean hourly values for 24 h in 5 middle-aged mice. Regression analysis comparing HF power and sleep–wake parameters during ManNAc treatment indicated that HF power was positively correlated with SWS. Moreover, there was a negative correlation between HF power and WAKE following ManNAc treatment (Fig. 4). However, there was no correlation between HF power and sleep–wake state in the control period.

## 4. Discussion

The purpose of this study was to determine if bio-behavioral activity and sleep–wake quality in middle-aged mice might improve with ManNAc treatment. These data are the first to show that ManNAc treatment in middle-aged mice improves bio-behavioral activity. This is predominantly due to differences in sleep–wake patterns present increase wakefulness time, and less time in SWS, during the dark phase. Second, a relationship between parasympathetic nervous activity and sleep–wake state is observed with ManNAc treatment. Finally, ManNAc enhanced diurnal amplitude of rhythm in most of the parameters. These results suggest that ManNAc could be used to improve bio-behavioral activity and sleep–wake patterns in middle-aged animals including humans.

To date, ManNAc is interested in a potential therapeutic agent for improving quality of life as well as cognitive function, because ManNAc treatment alleviated the age-related decline in learning ability and memory in both mice and dogs (Kikusui et al., 2012; Nagasawa et al., in press). ManNAc can rescue the sialylation of glycoproteins in GNE-knockout embryonic stem cells (Schwarzkoph et al., 2002), and

**Table 1**
Percent changes of bio-behavioral activity to ManNAc treatment in mice.

| Measure | Young | | Middle-aged | | Old | |
|---|---|---|---|---|---|---|
| | Light | Dark | Light | Dark | Light | Dark |
| HR | 99.1 ± 2.3 | 102.5 ± 3.4 | 85.9 ± 3.4* | 90.2 ± 1.8* | 102.1 ± 2.7 | 104.1 ± 2.6 |
| BP | 99.9 ± 3.8 | 101.6 ± 5.1 | 94.2 ± 2.5* | 95.9 ± 2.6* | 99.9 ± 2.4 | 101.6 ± 2.8 |
| LA | 100.5 ± 3.7 | 108.4 ± 4.9 | 104.9 ± 3.8 | 215.3 ± 6.8* | 93.1 ± 4.2 | 102.1 ± 3.4 |

HR: heart rate, BP: body temperature, LA: locomotor activity.
Young (n = 4), middle-aged (n = 5) and old (n = 3) mice. Data represent mean ± SEM.
* $P < 0.05$ significantly changed with ManNAc treatment.

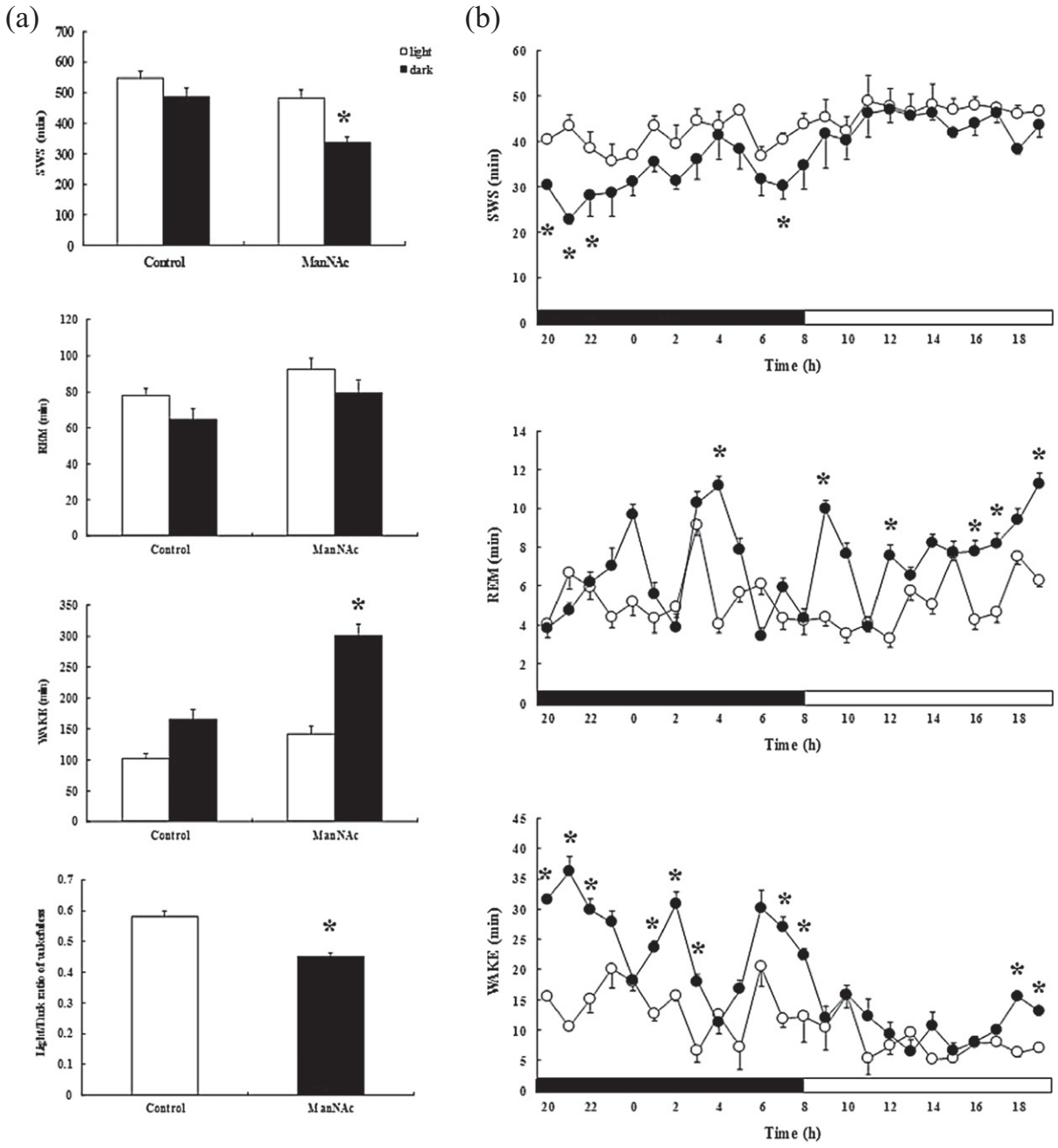


**Fig. 2.** Effects of ManNAc on sleep–wake state in mice. (a) The amount of slow wave sleep (SWS), rapid eye moving (REM) sleep and wakefulness (WAKE) during the 12-h light and dark phases, and light/dark ratio of wakefulness in control and ManNAc-treated middle-aged mice. Twelve-hour values for each mouse in each period are taken and averaged to get an average per mouse and then are summarized for 5 mice to get a mean ± SEM for each of the periods. *Significantly ($P < 0.05$) different from control values. (b) Hourly amount of SWS, REM sleep and WAKE for 24 h in control (open circle) and ManNAc-treated (filled circle) middle-aged mice. Data represent mean ± SEM (n = 5). *Significantly ($P < 0.05$) different from control values.

induce neural proliferation and dendrite outgrowth *in vitro* (Kontou et al., 2008). Moreover, GNE mutation was found in the patients of distal myopathy and this symptom was recovered by the treatment of ManNAc in drinking water (Malicdan et al., 2009). Present results are consistent with earlier observations as described above.

In mice, a decrease in the amplitude of the sleep–wake rhythm has been reported with age (Eleftheriou et al., 1975; Welsh et al, 1986). The age-related changes in sleep duration appear also to depend on genetic background in mice (Eleftheriou et al., 1975). Hasan et al. (2012) reported that in B6 mice, which are used in this study, total sleep time initially increases with age but then decreases in the old (2 years) mainly due to changes in sleep duration during the dark phase. SWS during the dark phase in middle-aged (1 year) is longer than that in young (3 months). Moreover, sleep fragmentation especially during the light phase in middle-aged is higher than young. Our results clearly showed that ManNAc could improve the diurnal amplitude of the wakefulness rhythm in middle-aged mice. Moreover, ManNAc could bring the values of sleep–wake state closer to those observed for young mice. We thought that ManNAc might induce a decrease of sleep fragmentation in the light phase, and this improvement of sleep quality might be related to the wakefulness during the dark phase. Because sleep duration during the dark phase in both young and old mice was shorter than that in middle-aged mice (Hasan et al., 2012), the effects of ManNAc were not observed in those aged mice. Furthermore,

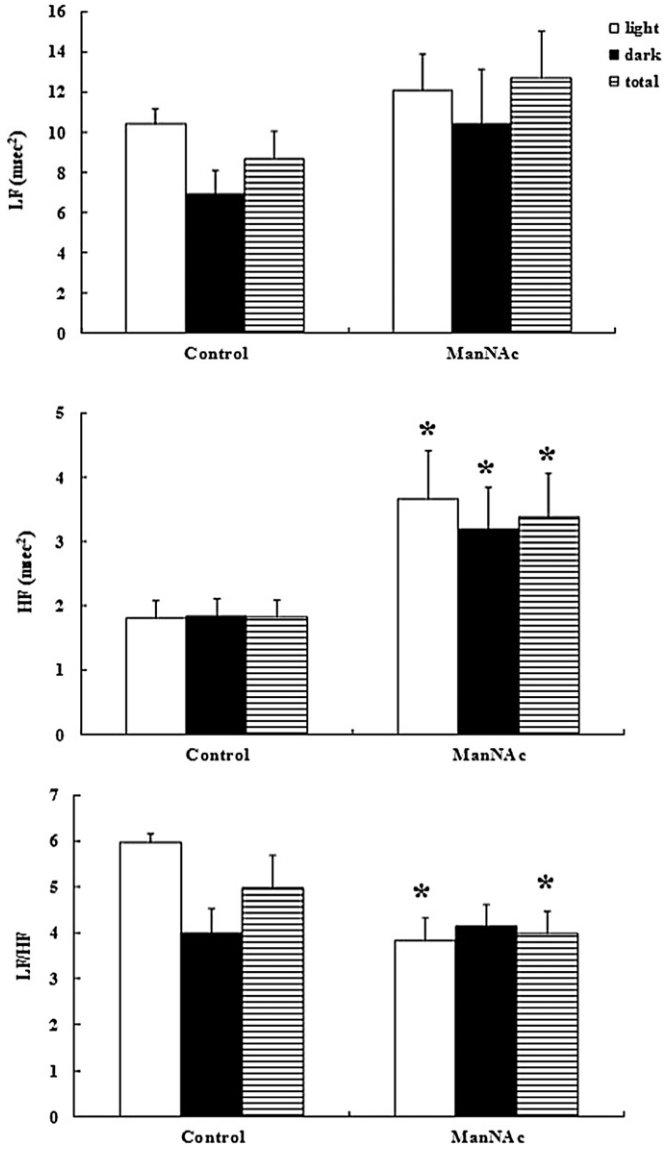


**Fig. 3.** Light- and dark-phase parameters in heart rate variability in control and ManNAc-treated middle-aged mice. Twelve-hour values for each mouse in each period are taken and averaged to get an average per mouse and then are summarized for 5 mice to get a mean ± SEM for each of the periods. *Significantly ($P < 0.05$) different from control values. LF: low frequency, HF: high frequency, and LF/HF: LF to HF ratio.

middle-aged mice might just reveal the beginning of age-associated changes, the efficacy of ManNAc could appear dramatically. It is widely assumed that many sleep parameters are changing with age in humans with sleep becoming gradually more fragmented and a reduction in day–night sleep differences (Bliwise, 1993; Carrier et al., 2001). ManNAc may play an important role for sleep disorder with aging in humans.

The spectral analysis of HRV is a noninvasive tool to quantify the relative amount of sympathetic and parasympathetic nervous activities. Generally, the HF power provides an index of parasympathetic nervous activity. Further, the LF/HF ratio is a convenient index of parasympathetic and sympathetic nervous interaction (Malik et al., 1996). Sympathetic nerve activity at rest is widely found to increase during aging (Hotta and Uchida, 2010). Although an impaired ability to adapt to environmental or intrinsic visceral stimuli in the elderly, there is less research on aging of the parasympathetic nervous system. However, age-related decline in vagus nerve function is suggested to be based on the findings that the vagal component of HRV decreases with age (Korkushiko et al., 1991) and that HR changes in response to muscarinic receptor blocking agents are blunted in age dependently (Poller et al., 1997). The autonomic nervous function in middle-aged mice was shifted sympathetic predominance during the light phase, because the LF/HF ratio was higher than that of the dark phase. Moreover, normal diurnal variations of the autonomic nervous activity disappeared in middle-aged mice. It might partly be dependent on the sleep quality such as an increased sleep fragmentation and a reduction in day–night sleep differences. To our knowledge, this is the first report to observe the impairment of autonomic nervous function in middle-aged mice.

Although the improvements of bio-behavioral and sleep quality are associated with ManNAc treatment, the mechanisms underlying these effects are unknown. We thought that autonomic nervous function especially parasympathetic nervous activity might be relevant to these phenomena, because responses on lowering HR and BT were observed after only a few days of treatment. Moreover, regression analysis clearly showed that parasympathetic nervous activity is correlated to the sleep–wake state. Because BT and sleep–wakefulness influence the counterpart in various extents (Kurachi and Deboer, 2010), modulating BT by changing autonomic nervous function might affect the sleep quality. Even though, the reason why ManNAc could repair the autonomic nervous function is still unknown. Further studies are needed to clarify the pharmacological properties of ManNAc including doses and length of treatment period.

Another possibility is the increase in orexin neurons. Neurons that express the neuropeptides orexins A and B were discovered in the late 1990s (de Lecea et al., 1998; Sakurai et al., 1998) and are located exclusively in the lateral hypothalamus and contiguous perifornical area.

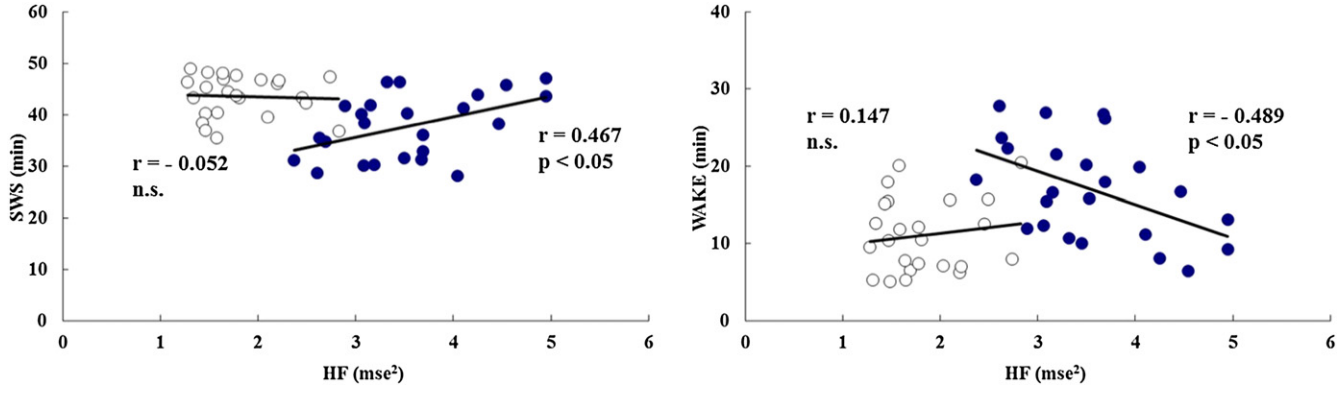


**Fig. 4.** Correlation between high frequency (HF) power and sleep–wake states in control (open circles) and ManNAc-treated (filled circles) middle-aged mice. Regression analysis was performed using mean hourly values of HF, slow wave sleep (SWS) and wakefulness (WAKE) for 24 h in 5 middle-aged mice. r = correlation coefficient, $P < 0.05$ = significant correlation, n.s. = not significant.

Orexin neurons appear to play a crucial role in sleep architecture and stabilization of state-dependent behavior (Mochizuki et al., 2004). Orexin regulates and consolidates sleep–wake patterns and has important roles in physical activity and energy homeostasis. Orexins also modulate food intake, consistent with their responsiveness to circulating factors indicative of metabolic status, such as glucose and leptin (Tsujino and Sakurai, 2009). Collectively, the broad range of functions influenced by the orexin system has led to the description of these neurons as "physiological integrators". Moreover, earlier studies have suggested an age-related decline in orexin function either at the expression or receptor level (Porkka-Heiskanen et al., 2004; Terao et al., 2004; Kessler et al., 2011). ManNAc treatment had a positive effect on neurogenesis in the hippocampus as well as on improved object recognition in middle-aged mice (Kikusui et al., 2012). In addition, ManNAc contributes to epigenetic modifications at the *Hcrt* gene locus to induce orexin neuron differentiation (Hayakawa et al., 2013). Therefore, it is possible that the improvement in bio-behavioral activity and sleep–wake state in middle-aged mice seen in this study might depend on an increase in orexin neurons due to the epigenetic modulatory activity of ManNAc. Actually we have observed that ManNAc treatment in middle-aged mice increased orexin neurons in the lateral hypothalamus (unpublished observation). However, the decline of neurogenesis in aged animals was recovered by physical activity or environmental enrichment (Kempermann et al., 1998; van Praag et al., 2005). Therefore, the improvement of sleep quality with ManNAc treatment might be mediated by increased LA or rhythmic bio-behavioral activity.

In conclusion, results in the present study have indicated that ManNAc can improve bio-behavioral and sleep quality in middle-aged mice. Of course further studies will be needed to clarify the mechanisms of improvement. ManNAc can be used to persons who have the sleep disorder narcolepsy, characterized by increased daytime sleepiness, sleep fragmentation, and low physical activity.

## Disclosure statement

None of the authors have any conflicts of interest.

## Acknowledgments

This work was supported in part by the Advanced Research for Medical Products Mining Program of the National Institute of Biomedical Innovation (NIBIO), Japan (09-1) and Gran-In-Aid from the Ministry of Education, Science, Sports and Culture, Japan (22580348).

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